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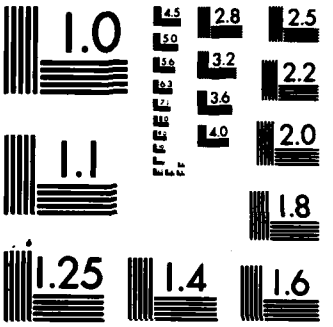

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THERAPEUTIC EFFECTIVENESS OF CRYOPRESERVED AUTOLOGOUS PLATELETS IN THE TREATMENT OF THROMBOCYTOPENIC DOGS

by

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Canine platelets were preserved in the frozen state with 6% DMSO in a -80 C mechanical freezer. The recovery of platelets after the freeze-thaw-wash procedure was about 70%, and posttransfusion recovery values were about 40% those of fresh platelets. The washed previously frozen platelets were hemostatically effective in supporting lethally irradiated dogs through prolonged periods of thrombocytopenia.

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INTRODUCTION

Following the successful cryopreservation of canine platelets with a glycerol-plasma solution (2), several other investigators used 5 to 6% DMSO as the cryoprotectant for freezing and storing human platelets at -80 C or -150 C, with successful results (4,5,7,8,10,11).

In our study reported here, canine platelets frozen with 6% DMSO and stored at -80 C were autotransfused to healthy dogs to make survival measurements, and to thrombocytopenic dogs that had received whole body irradiation to evaluate therapeutic effectiveness.

METHODS

Collection of Blood

Healthy non-splenectomized Beagle dogs, weighing 10 to 15 kg, were studied. All studies were performed without sedation or anesthesia.

On 13 separate occasions, a 200 ml sample of blood was collected in 30 ml of acid-citrate-dextrose (ACD) anticoagulant from each of 5 dogs for preparation of a platelet concentrate. Before blood collection, a 37.5 ml volume of the 67.5 ml volume of ACD anticoagulant (NIH, Formula A) was removed from the primary bag of the triple polyvinyl-chloride (PVC) plastic collection bag system (PL-146) (Fenwal Laboratories, Deerfield, IL); this was done to maintain a blood-to-ACD ratio of 6.7:1. After phlebotomy the dog was given about 150 ml of a 0.9% sodium chloride solution to maintain the blood volume.

Isolation of Platelets From a Unit of Blood

Using serial differential centrifugation with a Sorval RC-3 refrigerated centrifuge (DuPont Instruments, Newtown, CT), the platelet concentrate was prepared from the blood sample at 22 $\pm$ 2 C. The blood was spun at 800 g for 2.5 minutes, and a plasma expressor was used to remove the platelet-rich plasma (PRP) into one of the two transfer packs integrally attached to the primary collection bag. The concentrated red blood cells were returned to the dog. The PRP was centrifuged at 4500 g for 5 minutes to concentrate the platelets, and all but 30 ml of the platelet-poor plasma (PPP) was expressed from the platelet concentrate into the integrally attached transfer pack. The platelet

concentrate was kept undisturbed at room temperature for 30 minutes, after which it was resuspended gently by manual agitation. Before autotransfusion of the fresh platelets, they were labeled with ^{51}Cr so that posttransfusion survival could be measured.

Isolation of Platelets By Discontinuous-Flow Centrifugation Using the Haemonetics Blood Processor 30

Platelets were obtained from three dogs by plateletpheresis prior to treatment with 800 rads of whole body irradiation and were frozen for subsequent autotransfusion. Red cells and bone marrow also were cryopreserved prior to irradiation and were used subsequently to treat anemia, thrombocytopenia, and bone marrow aplasia.

The Haemonetics Model 30 discontinuous-flow Blood Cell Separator (Haemonetics Corp., Braintree, MA) was used for the plateletpheresis process. Platelet-rich plasma containing red blood cells was collected in a 125 ml pediatric bowl. A volume of ACD, Formula A, equivalent to 7.5% of the volume of the platelet-rich concentrate, was added and the mixture was centrifuged at 160 g for 10 minutes. The platelet-rich plasma now free of red blood cells was expressed into a transfer pack. The platelets were concentrated by centrifugation at 4500 g for 5 minutes, and all but 30 ml of the platelet-poor plasma was expressed from the platelet concentrate into another transfer pack. The platelet-poor plasma was frozen at -20 C for subsequent use. The platelet concentrate was kept undisturbed at room temperature for 90 minutes, and before cryopreservation was gently resuspended by manual agitation.

Cryopreservation of Dog Platelets

Platelets were isolated either from a unit of whole blood or from a healthy donor by plateletpheresis using the Haemonetics Blood Processor 30. A 30 ml volume of platelet concentrate was placed on a modified Eberbach shaker. A 30 ml volume of 12% DMSO-plasma solution, prepared prior to use by adding the DMSO slowly to the plasma to avoid heat production, was added to the platelet concentrate over 30 minutes with mixing at 180 lateral oscillations per minute. The resultant 60 ml volume of platelet-plasma-DMSO mixture was transferred to either a 200 cm^2 polyolefin plastic freezing container (Delmed) or a 300 ml PVC plastic transfer pack (Fenwal). This was placed in an aluminum container and frozen at a rate of 2 to 3 C per minute in a Harris -80 C mechanical freezer. The platelets were stored frozen at -80 C for as long as 7 months.

The frozen platelets were thawed in a 42 C water bath for about 2.5 minutes, after which 300 uCi of $Na_2{}^{51}CrO_4$ (Squibb Chromotope) was added. The mixture was incubated at room temperature for 30 minutes. The ^{51}Cr-labeled platelets used in 5 of the autotransfusions were washed by rapid dilution with 100 ml of a solution composed of 0.9% NaCl, 0.2% glucose, and 80 mg% inorganic phosphorus with a pH of 5.0. The ^{51}Cr-labeled platelets used in 4 other autotransfusions were diluted in a stepwise fashion, first with 30 ml of the sodium chloride-glucose-phosphate solution over a 20-minute period with agitation, followed by two successive 10-minute periods of equilibration with agitation, and then addition of the remaining wash solution over a 10-minute period. Both the rapidly diluted and slowly diluted platelet mixtures were centrifuged at 4500 g for

5 minutes. All the visible supernatant was removed, and the platelets were resuspended in 30 ml of sodium chloride-glucose-phosphate solution until the time of autotransfusion.

Platelet counts by phase microscopy were used to quantitate the freeze-thaw-wash recovery values. The platelet recovery in vitro was determined by dividing the total number of platelets surviving the freeze-thaw-wash procedure by the total number of platelets frozen.

Four healthy dogs received autotransfusions of fresh platelets labeled with 300 uCi of $Na_2{}^{51}CrO_4$. After addition of the ^{51}Cr to these platelets, they were incubated at room temperature for 30 minutes, and then were diluted rapidly with 100 ml of the sodium chloride-glucose-phosphate solution. After centrifugation at 4500 g for 5 minutes, all the visible supernatant was removed. The platelet concentrate was resuspended in 30 ml of the sodium chloride-glucose-phosphate solution until the time of autotransfusion.

Platelet survival measurements were made in normal dogs as described by Aster and Jandl (1). Samples were obtained before transfusion, 1 and 2 hours after transfusion, and daily for up to 7 days after transfusion (11). Blood volume was determined from the plasma volume measured with ^{125}I-labeled albumin and the total body hematocrit (9). The peripheral venous hematocrit was multiplied by 0.89 to calculate the total body hematocrit (3).

The lifespan of the platelets was estimated from the disappearance of radioactive ^{51}Cr from the recipient's circulation. The T_{50} value refers to the time (in days) in which 50% of the radioactivity present

2 hours after infusion is removed from the circulation.

Therapeutic Transfusion of Cryopreserved Platelets

A total of 22 autologous transfusions of washed previously frozen platelets were studied in three lethally irradiated thrombocytopenic dogs at a time when platelet counts were less than 10,000/mm^3. The platelet concentrates had been prepared and frozen before the dogs were treated with 800 rads whole body irradiation.

Following irradiation, the dogs were placed in a clean room and treated with fluid by clysis, and with oral and parenteral antibiotics. In addition to the autologous cryopreserved platelets, the dogs also were given autologous transfusions of washed previously frozen red blood cells and 1-4 X 10^9 washed previously frozen autologous bone marrow cells to repopulate their bone marrow (Figures 1-3).

Platelet recovery in vivo was determined as follows:

$$\% \text{ Recovery} = \frac{(\text{Plt Ct posttransfusion} - \text{Plt Ct pretransfusion})}{\text{Total No. Plts Transfused}} \times 10^3 \times \text{Blood Volume (ml)}$$

Total blood volume was measured prior to irradiation. The therapeutic effectiveness of the cryopreserved platelets was assessed by clinical bleeding and occult blood in the stool.

RESULTS

The cryopreserved platelets prepared from individual units of whole blood had freeze-thaw-wash recovery values of 67 ± 20% (n = 5) when washed by the rapid-dilution method, and values of 65 ± 19% (n = 4) when washed by the slow-dilution method. Plateletpheresed cryopreserved platelets, isolated by discontinuous-flow centrifugation in the Haemonetics Model 30 Blood Processor, had freeze-thaw-wash recovery values of 71 ± 17% (n = 22) whether the rapid-dilution or slow-dilution wash method was used.

Studies of a total of 22 autotransfusions of washed previously frozen platelets to three thrombocytopenic dogs showed a mean in vivo recovery value of 30 ± 17% (n = 22). The responses to the platelet autotransfusions in these dogs are shown in Figures 1-3. Platelet counts increased in relation to the number of platelets infused. Results were similar whether the platelets were isolated from units of whole blood or isolated by plateletpheresis by discontinuous-flow centrifugation with the Haemonetics Model 30, and whether the platelets were frozen in polyolefin plastic bags or in PVC plastic bags.

FIG. 1
FIG. 2
FIG. 3A
FIG. 3B

Platelet lifespans assessed by platelet counts in thrombocytopenic dogs were shorter than platelet lifespans measured by a ^{51}Cr labeling procedure in normal dogs.

None of the three thrombocytopenic dogs supported with 22 autotransfusions of washed previously frozen platelets had any episodes of hemorrhage or sign of occult blood in the stools. In two of the dogs,

washed previously frozen platelets were autotransfused until bone marrow reconstitution was complete; the third dog died of sepsis while still leukopenic.

During the first 2 hours after autotransfusion of fresh ^{51}Cr-labeled platelets to normal dogs, recovery values were about 82%; the lifespan was about 8 days with a linear removal rate and a T_{50} value of about 4 days (Figure 4). When platelet concentrates isolated from units of blood were frozen with 6% DMSO and stored at -80 C, thawed, washed by either the slow or the rapid dilution procedure, and autotransfused to normal dogs, in vivo recovery values were about 33%; the lifespan was about 8 days with a linear removal rate and a T_{50} of about 3 days (Figure 4). There was an insignificant difference ($p > 0.1$) in the mean in vivo recovery value between platelets washed by the rapid dilution method ($36 \pm 11\%$, $n = 5$) and those washed by the slow dilution method ($28 \pm 10\%$, $n = 4$). FIG. 4

DISCUSSION

Canine platelets have been frozen with 6% DMSO and storage at -80 C with satisfactory results. In vitro freeze-thaw-wash recovery values were similar to values reported from studies of human and baboon platelets frozen in a similar manner, but in vivo recovery values were lower than those reported for human and baboon platelets frozen in a similar manner (6,10). The thawed canine platelets in the study reported here were washed with a phosphate-buffered sodium chloride-glucose solution instead of the DMSO-ACD-plasma solution that has been used to wash human and baboon platelets (6,10), and this may explain our lower in vivo survival values.

Kim and Baldini (4) have reported improved posttransfusion survival values when the plasma wash solution was added gradually to thawed human platelets, but we observed no significant differences in in vitro or in vivo recovery values whether the wash solution was added gradually or rapidly to the previously frozen dog platelets.

The transfusion of autologous washed previously frozen platelets produced acceptable increases in platelet counts in thrombocytopenic dogs and maintained normal hemostasis. The lifespan of the transfused platelets was shorter in the thrombocytopenic dogs than in normal dogs, and this shortened lifespan was associated with fever, leukopenia, and sepsis.

Canine platelets frozen with 6% DMSO at -80 C and washed before autotransfusion to thrombocytopenic dogs increased platelet counts and maintained hemostasis.

SUMMARY

Canine platelets were preserved in the frozen state with 6% DMSO in a -80 C mechanical freezer. The recovery of platelets after the freeze-thaw-wash procedure was about 70%, and posttransfusion recovery values were about 40% those of fresh platelets. The washed previously frozen platelets were hemostatically effective in supporting lethally irradiated dogs through prolonged periods of thrombocytopenia.

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FIGURE 1

Clinical course of lethally irradiated dog beagle #6 treated with autologous and homologous washed previously frozen blood components during marrow reconstitution.

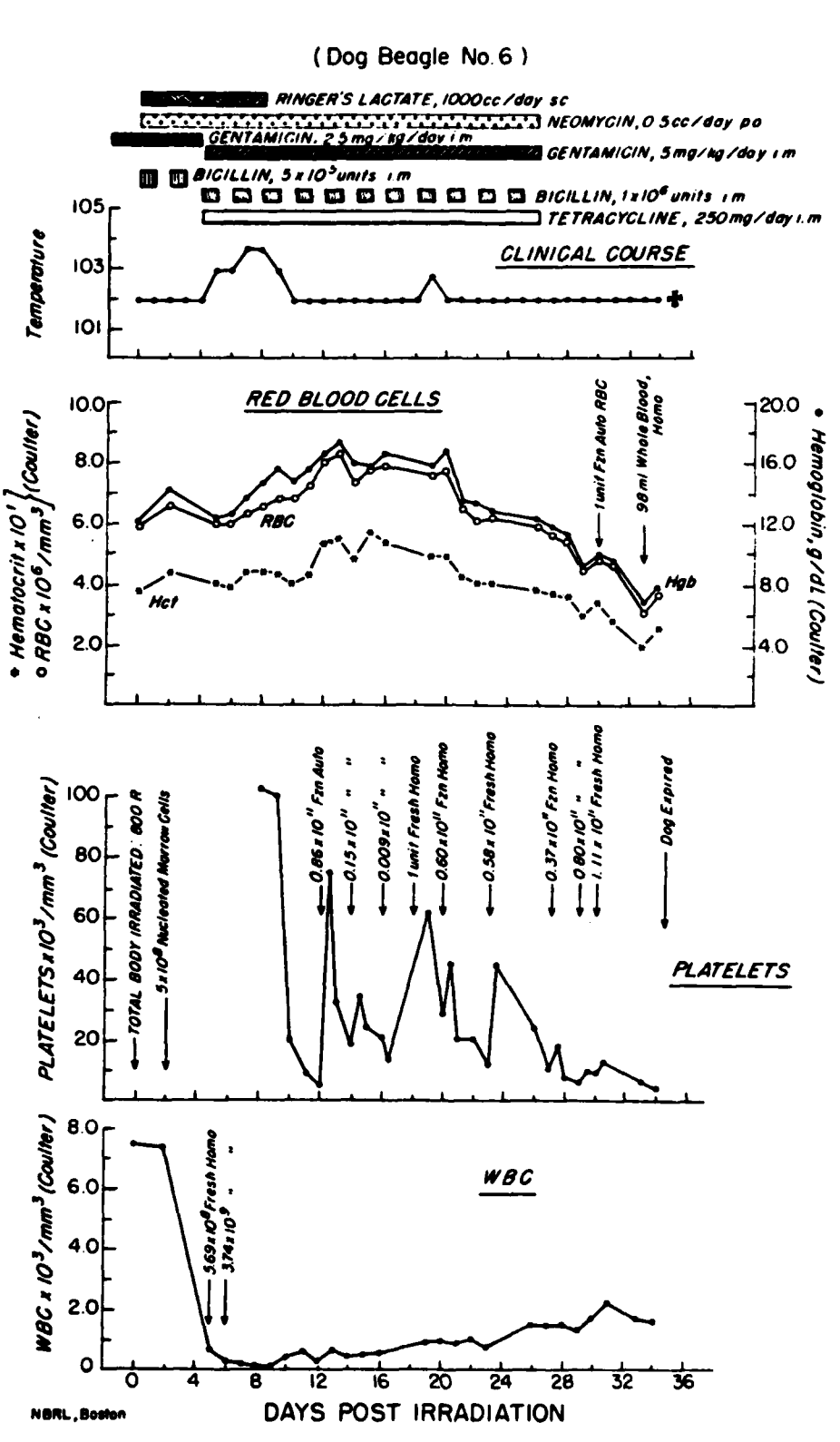

FIGURE 1

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FIGURE 2

Clinical course of lethally irradiated dog beagle #8 treated with autologous washed previously frozen blood components during marrow reconstitution.

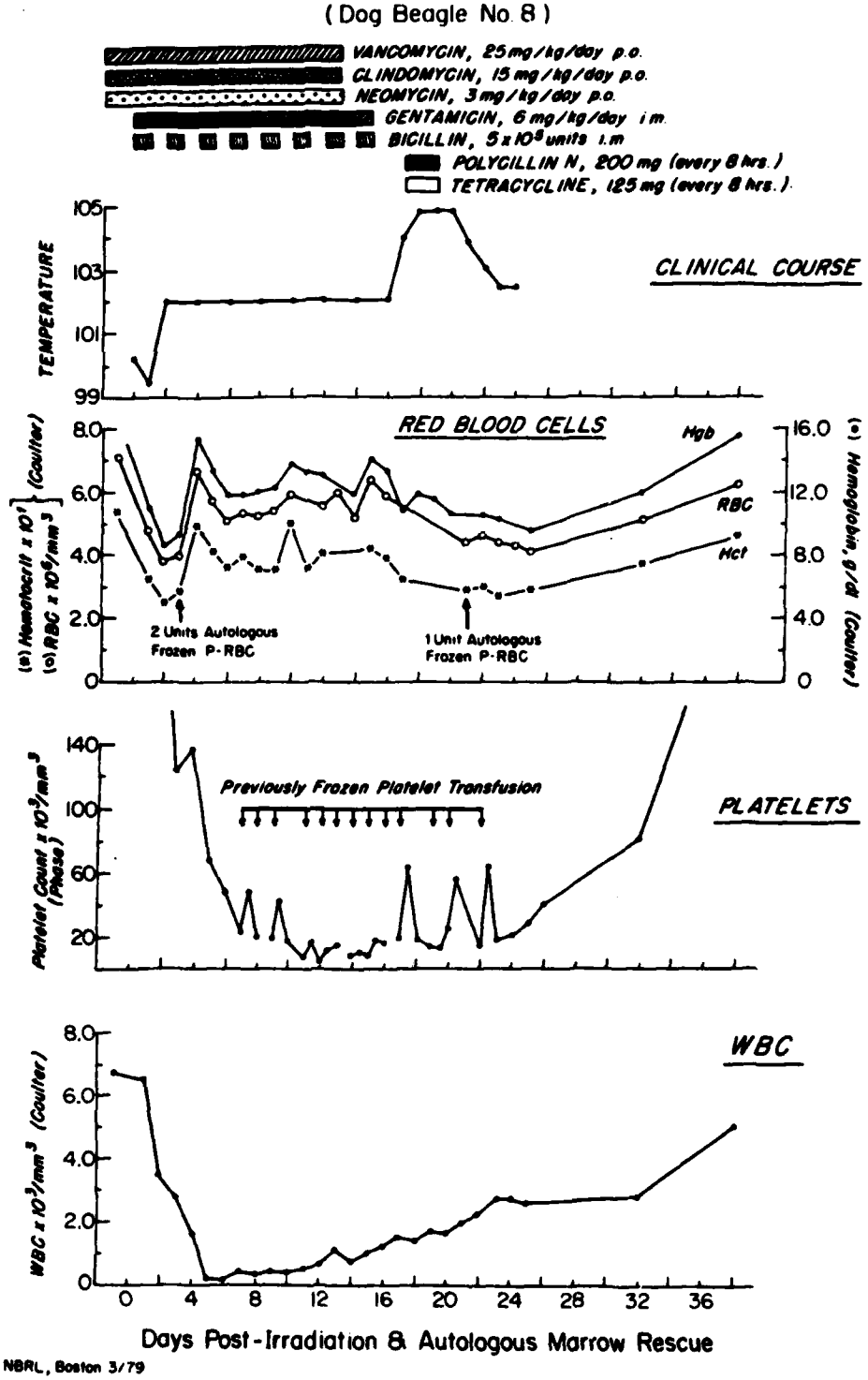

FIGURE 2

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FIGURE 3A

Clinical course of lethally irradiated dog Beagle #10 treated with autologous washed previously frozen blood components during marrow reconstitution.

(Dog Beagle No. 10)

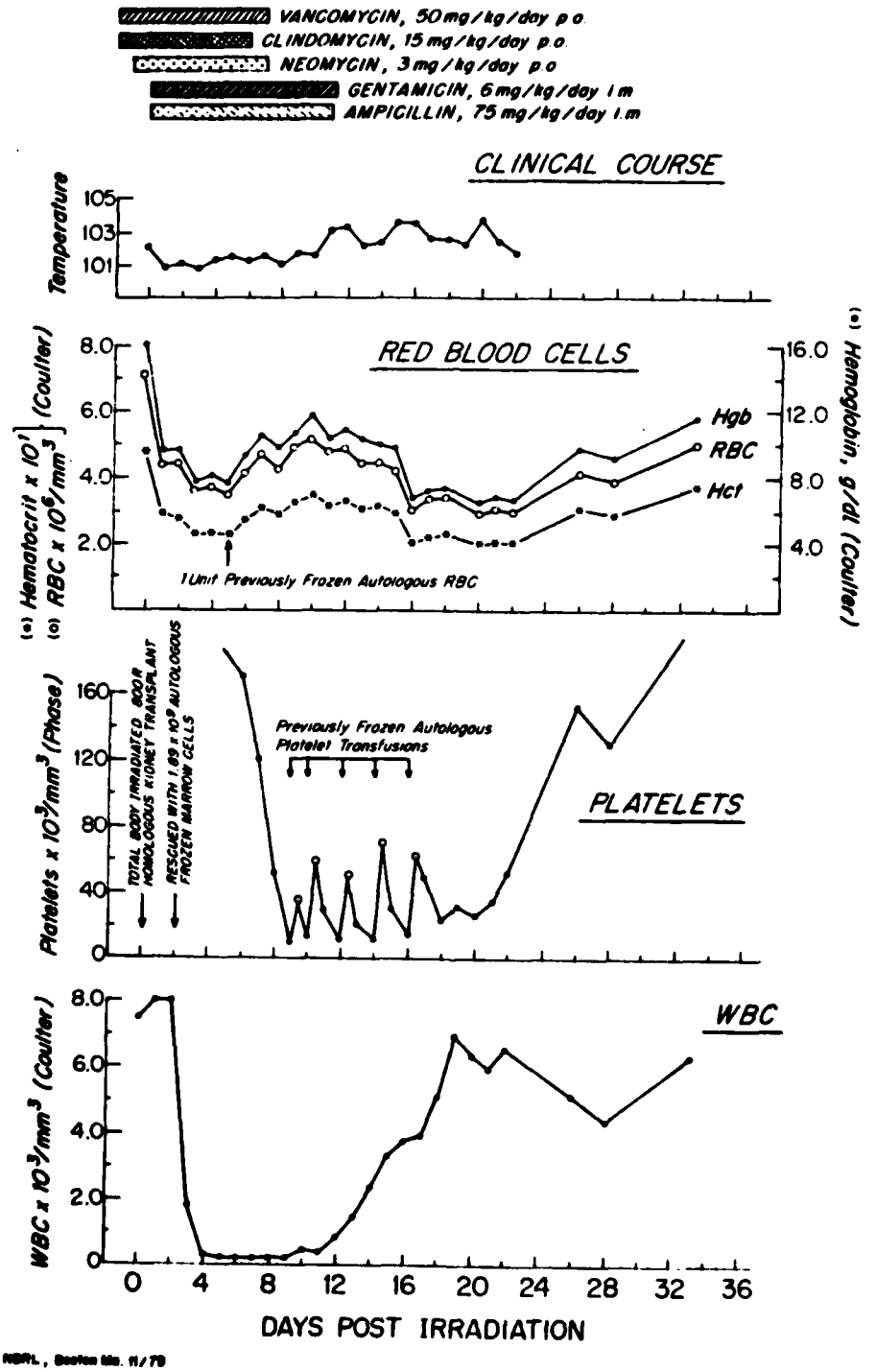

FIGURE 3A

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FIGURE 3B

Platelet counts in a lethally irradiated dog after transfusion of washed previously frozen platelets.

FIGURE 3B

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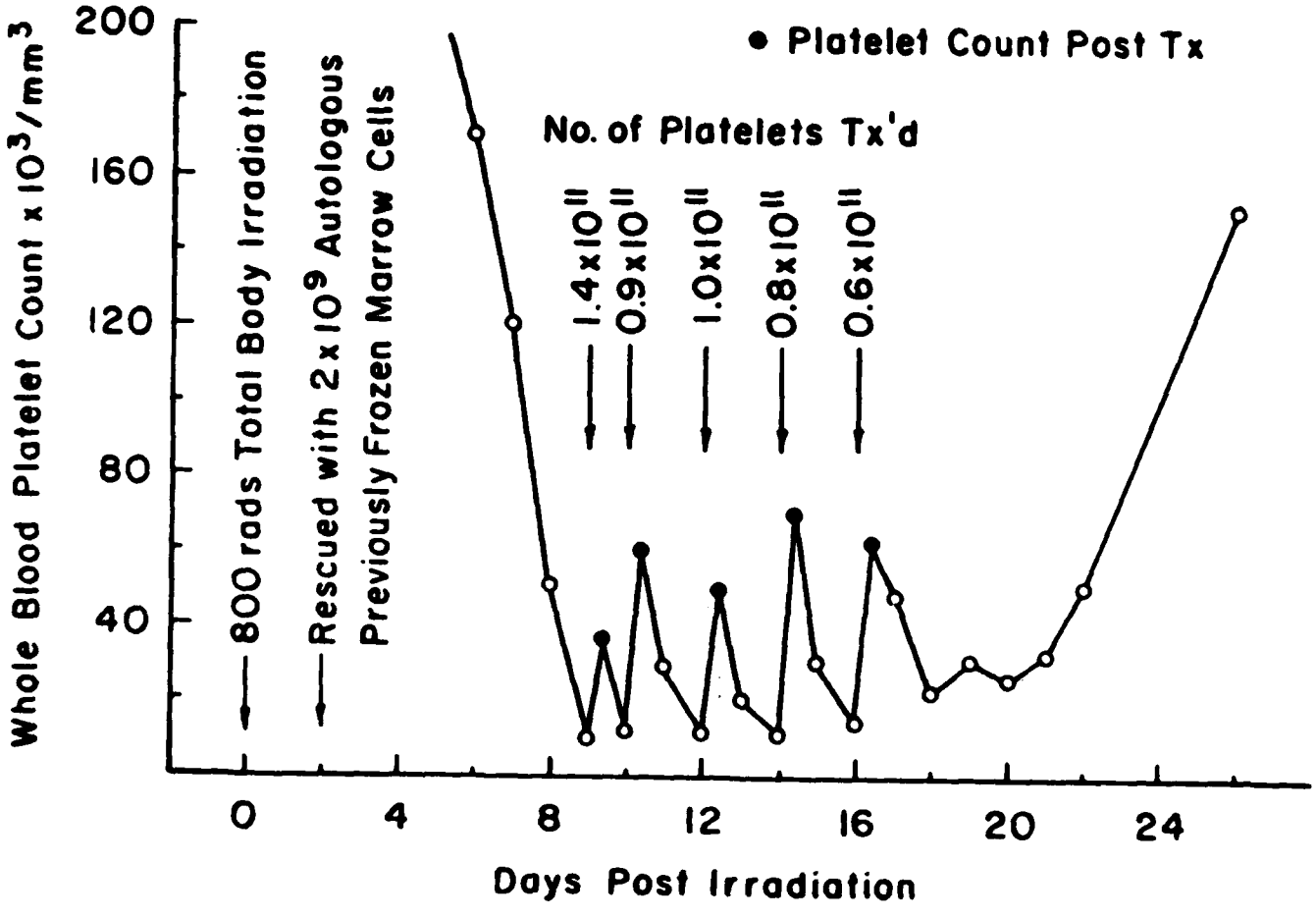

FIGURE 4

^{51}Cr survival values of canine autologous transfusions of fresh platelets and platelets frozen with 6% DMSO at -80 C and washed prior to autotransfusion.

FIGURE 4

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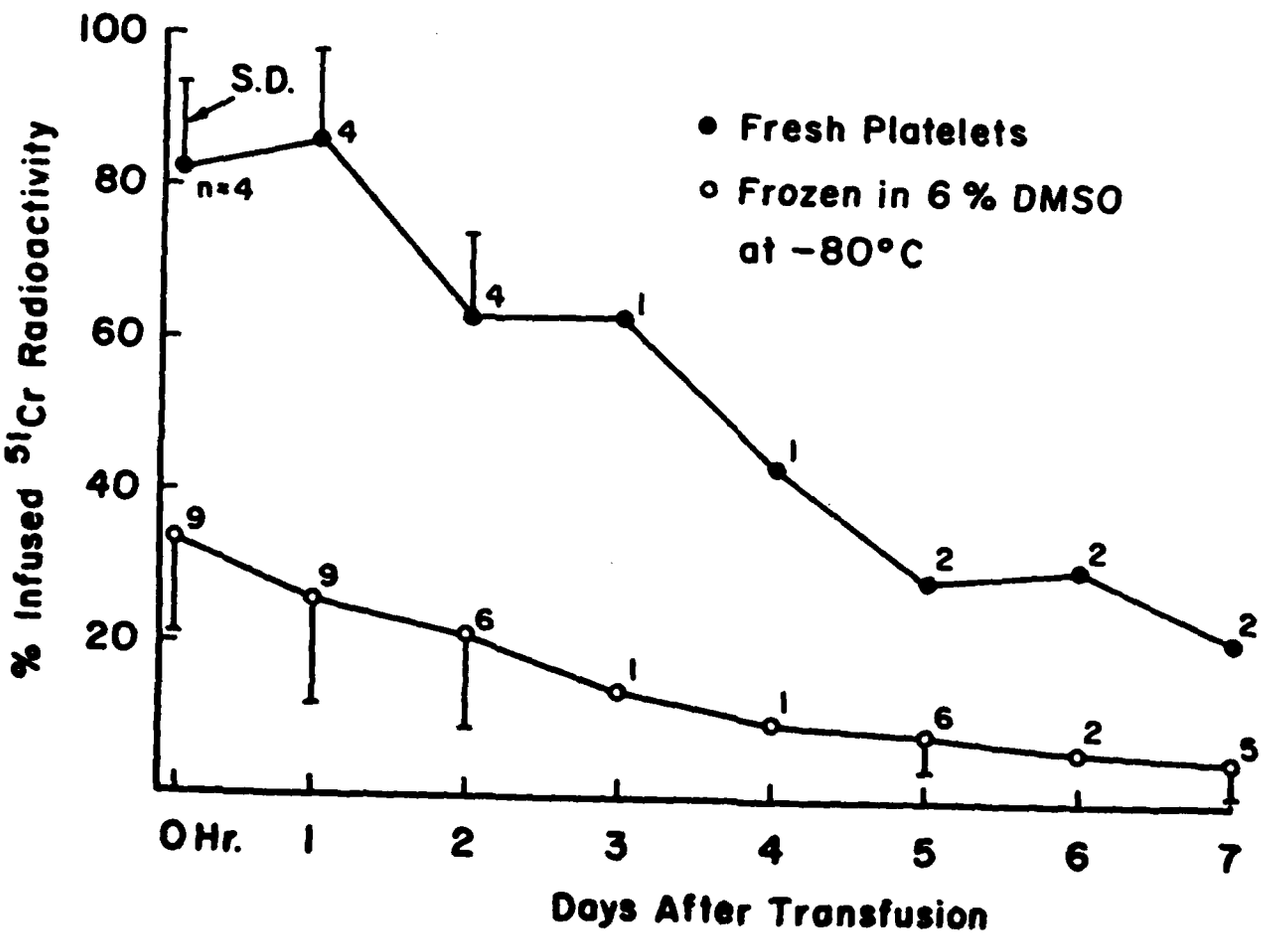

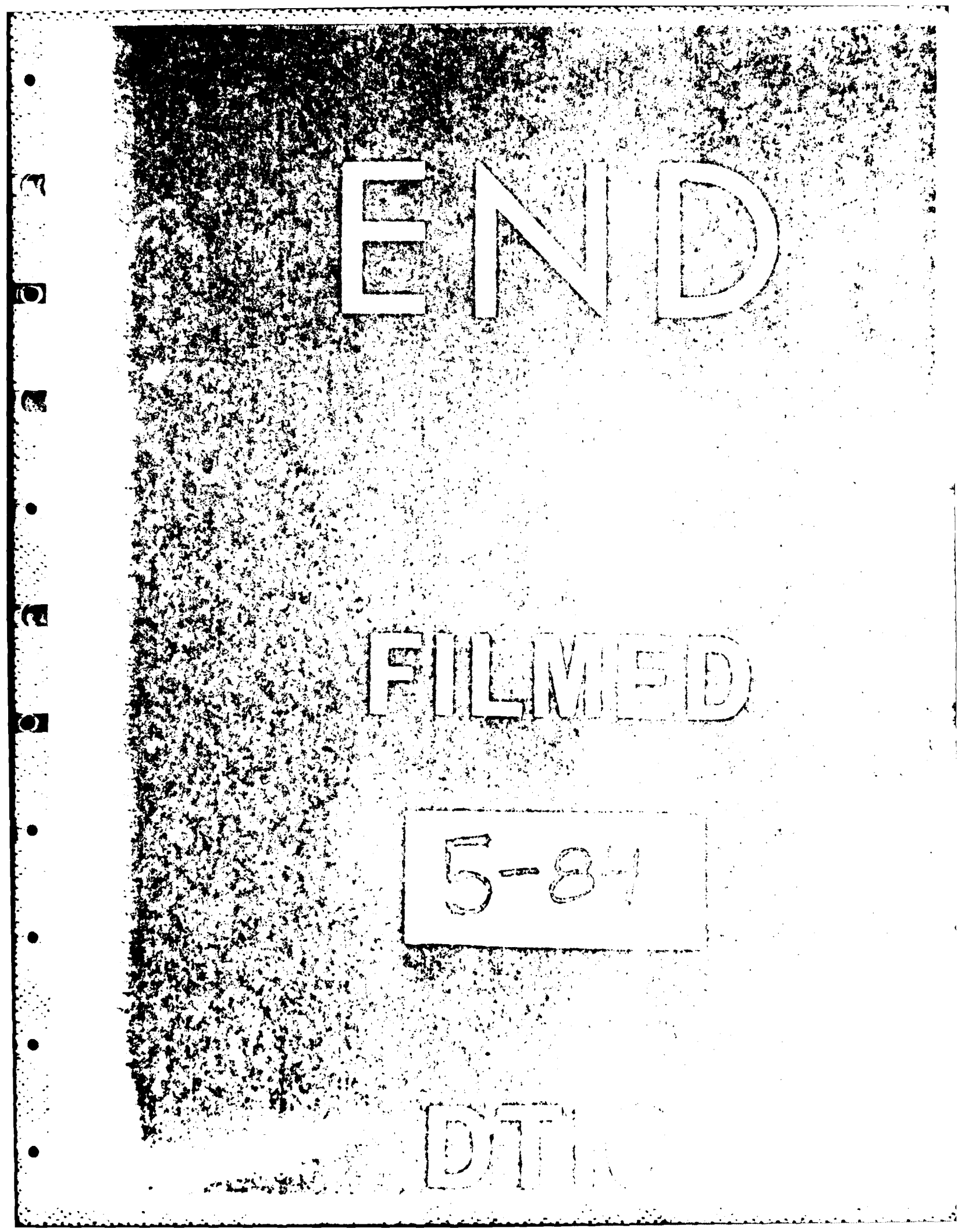
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